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Are Argyrophilic Nucleolar Organizer Regions Good Prognostic Indicators of Survival of Patients With Esophageal Cancer With Lymph Node Metastasis?

MASAHIDE IKEGUCHI, MD, KUNIYUKI KATANO, MD, ATSUO OKA, MD, SHUNSUKE SHIBATA, MD, SHUNICHI TSUJITANI, MD, MICHIO MAETA, MD, AND NOBUAKI KAIBARA, MD

From the Department of Surgery I, Faculty of Medicine, Tottori University, Yonago, Japan

Argyrophilic nucleolar organizer regions (AgNORs) were evaluated in 95 samples from primary esophageal squamous cell carcinomas and 75 samples from metastatic lymph nodes. The number of AgNORs per nucleus in primary tumors with positive nodes (n = 53, 6.1 ± 1.8) was greater than that in primary tumors with negative nodes (n = 42, 3.8 ± 1.1, $P < 0.001$). In 39 of 53 patients with positive nodes, the numbers of AgNORs per nucleus in metastatic lymph nodes were lower than those in primary tumors. The 5-year survival rate of these patients was 23.7%. However, the numbers of AgNORs per nucleus in metastatic lymph nodes were greater than those of primary tumors in 14 of 53 patients with positive nodes, and 11 of these 14 patients died from recurrence of cancer within 3 years after surgery. These observations suggest that the proliferative activity of cancer cells might be suppressed in the regional lymph nodes. However, cancer cells with higher proliferative activity in the regional lymph nodes than in the primary tumors might overcome immunological defenses and subsequent further metastasis might occur.

KEY WORDS: AgNORs, lymph node metastasis, 5-year survival, esophageal cancer

INTRODUCTION

Nucleolar organizer regions (NORs) are loops of ribosomal DNA (rDNA) located on the short arms of the acrocentric chromosomes 13, 14, 15, 21, and 22 [1–3]. These NORs are associated with transcription of rRNA and, thereby, with the regulation of protein synthesis. Increased expression of NORs appears to reflect cellular and nuclear activity. The silver-staining technique allows identification of nonhistone nuclear proteins associated with sites of rRNA transcription. Since the recent development of a modified version of the silver-staining technique, the identification of argyrophilic NORs (AgNORs) has been applied to sections of routinely processed paraffin-embedded tissue [2]. There is considerable evidence to suggest that numbers of AgNORs are correlated with the proliferation and differentiation of cells, in particular from studies by flow cytometry and by immunostaining of Ki-67 [4–6]. Previous studies have suggested that the numbers of AgNORs per nucleus might be a predictor of malignant potential or a marker of carcinoma in breast cancer [7], gastric cancer [8], and colon cancer [9]. In esophageal cancer, many reports have indicated that the number of AgNORs is a good indicator of malignant potential. Moreover, the proliferative activity of cancer cells of such primary tumors has been reported to be higher in cases with lymph node metastasis than in the cases without such metastasis [10,11].

The proliferative activity of cancer cells in the metastatic lymph nodes in cases of esophageal cancer has not

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Address reprint requests to Dr. Masahide Ikeguchi, Department of Surgery I, Faculty of Medicine, Tottori University, Nishimachi 36-1, Yonago 683, Japan.

yet been reported. It remains unclear whether cancer cells that metastasize to regional lymph nodes from the primary tumor in the esophagus display high proliferative activity in the lymph nodes. It is also unclear whether the AgNORs in primary tumors in the esophagus are still a good prognostic indicator for patients with esophageal cancer with lymph node metastasis.

In the present study, we analyzed the mean numbers of AgNORs per nucleus in cells from 95 samples of primary tumors and from 75 samples of metastatic lymph nodes for 95 patients with operated esophageal cancer. We examined whether the proliferative activity of cancer cells in the primary tumors and in the metastatic lymph nodes might be a good prognostic indicator for patients with esophageal cancer with lymph node metastasis.

PATIENTS AND METHODS

Clinical Material

Between 1981 and 1993, 121 patients with primary squamous cell carcinoma of the esophagus underwent esophagectomy at the Department of Surgery I, Faculty of Medicine, Tottori University Hospital. For the present evaluation of the correlation between the proliferative activity of cancer cells and the occurrence of lymph node metastasis or postoperative survival, patients who had undergone preoperative chemotherapy or radiation therapy were excluded from this study. In patients who had undergone preoperative chemotherapy or radiation therapy, there was a possibility that the proliferative activity of cancer cells might have been changed from that in the untreated condition. The 95 patients included 83 males and 12 females and ranged in age from 45 to 81 years. Each patient underwent total or subtotal esophagectomy with right thoracotomy and laparotomy, with mediastinal, abdominal, and, in some cases, cervical lymphadenectomy. There were no distant metastases at surgery. The resected specimens were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (4 μm thick) were stained with hematoxylin and eosin (H&E) for histopathological diagnosis.

Staining of AgNORs

Staining was performed by the method of Ploton et al. [2]. Tissue was fixed in 10% formalin and processed routinely in paraffin wax. Paraffin sections (4 μm) were cut and dewaxed in xylene and hydrated through a graded ethanol series. Two volumes of a 50% solution of silver nitrate (Wako Pure Chemical Industries, Ltd., Osaka) in water were added to one volume of 2% gelatin staining solution that contained 1% formic acid (Mutô Pure Chemicals, Ltd., Tokyo). The tissue sections were incubated with freshly prepared AgNOR-staining solution for 30 minutes in darkness at room temperature. After rinsing with deionized water, the sections were dehydrated through a graded ethanol series to xylene. The stained specimens were examined with an oil immersion lens at a magnification of ×1,000. The AgNORs appeared as dots within the nuclei of cells. Five areas of the invasive margin from each sample were selected and at least 100 cells from each area (total over 500 cancer cells for each sample) were examined by a single observer who was ignorant of the outcome of each patient. For each specimen, the average number of AgNOR dots per nucleus (AgNOR score) was calculated.

Statistical Analysis

Pathological evaluations were based on the TNM classification proposed by the International Union Against Cancer in 1987 [12]. The chi-square test and Fisher's exact probability test were used to compare frequencies. Means were compared by Student's *t*-test. Five-year survival rates were calculated by the Kaplan–Meier method and the generalized Wilcoxon test was used to evaluate the statistical significance of differences. *P*-values of <0.05 were considered statistically significant.

RESULTS

AgNOR Scores and Clinicopathological Features

The AgNOR scores of primary tumors were closely related to the depth of tumor penetration (Table I). With the progression of primary tumors, AgNOR scores of primary tumors increased. The AgNOR scores of 95 samples from primary tumors and 75 samples of metastatic lymph nodes are shown in Table II. The mean AgNOR score of primary tumors from 53 patients with lymph node metastasis was significantly higher than that of primary tumors from 42 patients without lymph node metastasis ($P < 0.001$). The mean AgNOR score of 75 samples from metastatic lymph nodes was lower than that from primary tumors of 53 patients with lymph node metastasis. However, there was no statistically significant difference in the AgNOR scores of metastatic lymph nodes located at group 1 and 2 (close to the primary tumor) and that of metastatic lymph nodes located at group 3 (far from the primary tumor) [13]. The staining of AgNORs in a primary esophageal tumor without lymph node metastasis, in a primary esophageal tumor with lymph node metastasis, and in a metastatic lymph node are shown in Figure 1A–C.

Relationship Between the AgNOR Score and the Prognosis for Patients With Operated Esophageal Carcinoma

Fourteen patients who died from operative complications within 30 days after operation or who were lost to follow-up soon after operation were excluded from this part of the study. The 5-year survival rate of 32 patients with negative nodes was 73.9%, and this rate was significantly higher than that of 49 patients with positive nodes (17.7%, $P < 0.01$) (Fig. 2).

TABLE I. AgNOR Scores and Clinicopathological Features*

Depth of tumor invasion	No. of cases	No. of cases with metastatic lymph node (%)	AgNOR score or primary tumors (mean ±SD)
Tis and T1	23	3 (13)	4.0 ± 1.5[a]
T2	15	7 (47)	4.0 ± 1.4[b]
T3	32	20 (63)	5.6 ± 1.8[c]
T4	25	23 (92)	6.0 ± 2.0[d]
Total:	95	53 (56)	5.1 ± 1.9

*Tis, carcinoma in situ; T1, tumor has invaded the lamina propria or submucosa; T2, tumor has invaded the muscularis propria; T3, tumor has invaded adventitia; T4, tumor has invaded adjacent structures. SD, standard deviation. There are statistically significant differences between b and c ($P = 0.002$). However, there are no statistically significant differences between a and b ($P = 0.995$) or between c and d ($P = 0.451$).

We analyzed whether or not the AgNOR score could be a good prognostic factor independent of the lymph node metastasis. The 32 surviving patients without lymph node metastasis were divided into two groups according the AgNOR score of the primary tumor. The cutoff between the two groups was based on the mean AgNOR score of primary tumors without positive node (Table II). The 5-year survival rate of patients in the low-AgNOR group (AgNOR score <3.8, n = 19) was 84.2% and that of patients in the high-AgNOR group (AgNOR score ≥3.8, n = 13) was 54.3%. No statistically significant difference was observed ($P = 0.395$). Also, the 49 surviving patients with positive nodes were divided into two groups according to the AgNOR score of the primary tumor. The cutoff between the two groups was based on the mean AgNOR score of primary tumors with positive node (Table II). The 5-year survival rate of patients in the low-AgNOR group (AgNOR score <6.1, n = 31) was 16.6% and that of patients in the high-AgNOR group (AgNOR score ≥6.1, n = 18) was 20%. No significant difference was observed ($P = 0.379$) (Fig. 3). These 49 patients with positive nodes were also divided into two groups according to the AgNOR scores of the metastatic lymph nodes. The cutoff between the two groups was based on the mean AgNOR score of metastatic lymph nodes (Table II). The 5-year survival rate of patients in the low-AgNOR group (AgNOR score <5.3, n = 30) was 16.7% and that of patients in the high-AgNOR group (AgNOR score ≥5.3, n = 19) was 21.6%. No statistically significant difference was observed ($P = 0.211$) (Fig. 3).

When the AgNOR scores of primary tumors and those of metastatic lymph nodes were compared in 53 patients with positive nodes, the AgNOR scores of metastatic lymph nodes were higher than that of primary tumors in 14 cases (26.4%). In these 14 cases, one patient died from postoperative complications within 30 days after operation and 11 patients died from cancer recurrence within 3 years after surgery. Only 2 patients were alive at 11 and 13 months after operation (Fig. 4). The AgNOR scores of metastatic lymph nodes were lower than that of primary tumors in 39 cases (73.6%). In these 39 cases, 3 patients died from postoperative complications within 30 days after operation. The 5-year survival rate of the 36 surviving patients was 23.7%, and two patients were still alive more than 5 years after operation (Fig. 4).

DISCUSSION

The prognosis of patients with esophageal cancer is very poor. The 5-year survival rate of all patients with operated esophageal cancer is only about 30% [14]. Many patients died from recurrence, such as lymph node metastasis or hematogenic metastasis, soon after operation. Lymph node metastasis at operation appears to be one of the major factors in the poor prognosis [14,15].

The number of AgNORs per nucleus has recently been introduced as a good marker of the proliferative activity

TABLE II. Comparison of AgNOR Scores of Cancer Cells From Primary Tumors and From Metastatic Lymph Nodes in 95 Patients With Esophageal Cancer

		No. of cases	AgNOR score (mean ±SD)
Primary tumors	Without lymph node metastasis	42	3.8 ± 1.1[a]
	With lymph node metastasis	53	6.1 ± 1.8[b]
	Total:	95	5.1 ± 1.9[c]
Metastatic lymph nodes	Located in the group 1 and 2 lymph node (close to the primary tumor)	44	5.2 ± 1.7[d]
	Located in the group 3 lymph node (far from the primary tumor)	31	5.3 ± 2.1[e]
	Total	75	5.3 ± 1.9[f]

*SD, standard deviation. The nodal station was classified according to the "Guidelines for Clinical and Pathologic Studies on Carcinoma of the Esophagus" [13]. There are statistically significant differences between a and b ($P < 0.001$), and between b and f ($P = 0.0189$). However, there is no statistically significant differences between d and e ($P = 0.85$).

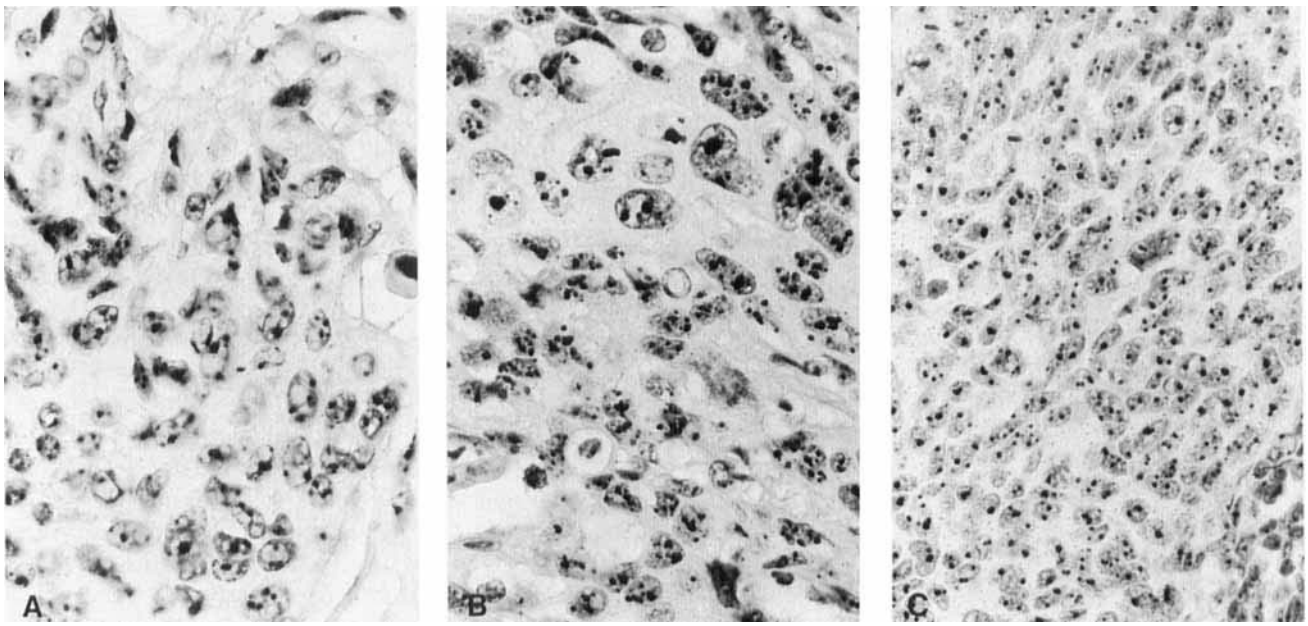

Fig. 1. **A:** Staining of AgNORs in esophageal carcinoma. This patient did not have lymph node metastasis, and the AgNOR score was 3.9. ×400. **B:** Staining of AgNORs in esophageal carcinoma. This patient had lymph node metastasis, and the AgNOR score was 7.3. ×400. **C:** Staining of AgNORs in a metastatic lymph node. ×200.

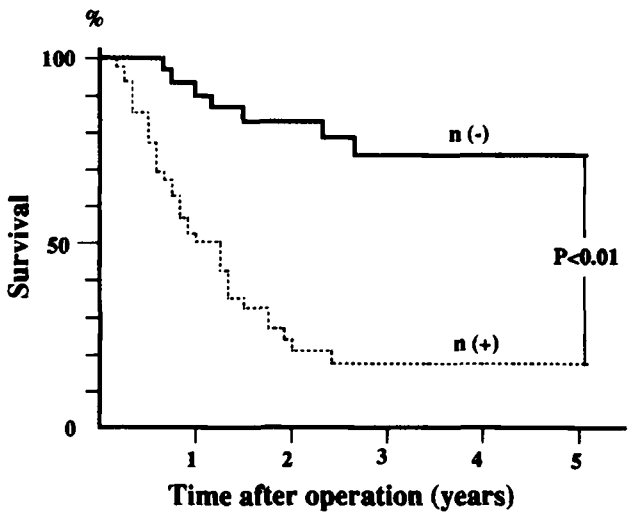

Fig. 2. Five-year survival rate of 32 patients with negative nodes (——) was 73.9% and that of 49 patients with positive nodes (. . .) 17.7%. There is a statistically significant difference between these two survival curves ($P < 0.01$).

of various tumors [5–9]. Morita et al. [10] reported that numbers of AgNORs per nucleus were closely correlated with lymph node metastasis, the depth of penetration, and the length of the tumor. Moreover, the prognosis of patients with larger numbers of AgNORs per nucleus was significantly poorer than that of patients with smaller numbers of AgNORs. Yoshida and colleagues [11] analyzed AgNORs in 92 patients with curatively resected esophageal carcinoma and reported that the rate of distant recurrence was significantly higher in patients with an AgNOR score of ≥4 than in those with an AgNOR score of <4. These results indicate that cancer cells in advanced esophageal cancer may have high proliferative activity and that AgNORs are a good marker of lymph node metastasis and a good prognostic indicator in patients with esophageal cancer.

However, it is unclear whether AgNORs are a good prognostic indicator in patients with esophageal cancer independent of the lymph node metastasis. It is also unclear whether or not cancer cells that metastasize to the regional lymph nodes still have high proliferative activity. We find no answers to these questions in the literature.

In this study, we analyzed the AgNOR scores of cancer cells in primary esophageal tumors and those of cancer cells in metastatic lymph nodes. The AgNOR scores of primary tumors were significantly higher in node positive than in node-negative tumors. However, in cases without lymph node metastasis, the 5-year survival rate of patients who had high-AgNOR scores in primary tumors did not differ from that of patients who had low AgNOR scores. In cases with lymph node metastasis, the 5-year survival rate of patients with high-AgNOR scores in primary tumors was almost the same as that of patients with low AgNOR scores. Moreover, in node positive cases, the 5-year survival rate of patients with high AgNOR scores in the metastatic lymph nodes was very similar to that of patients with a low AgNOR scores in metastatic lymph nodes. When we compared the proliferative activity of

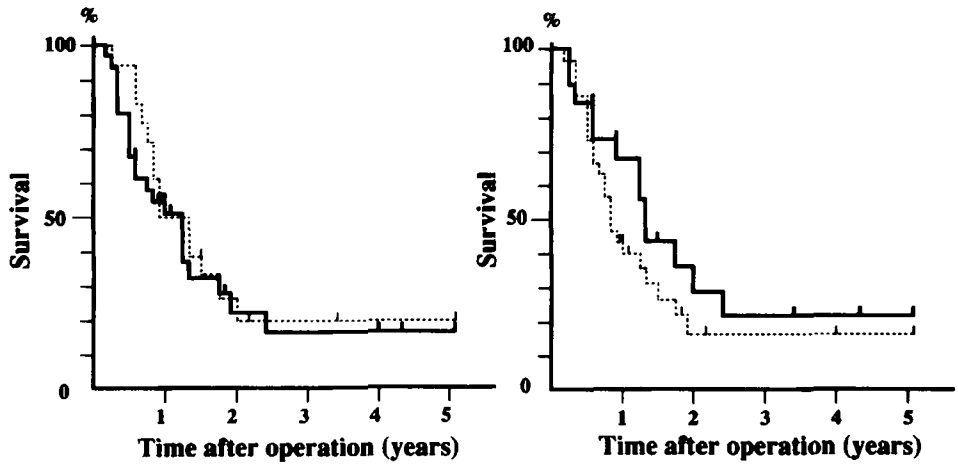

Fig. 3. Survival curves of patients with esophageal cancer with lymph node metastasis. **Left:** Patients were divided into two groups according to the mean AgNOR score of the primary tumor. The 5-year survival rate of patients in the low-AgNOR group (AgNOR score < 6.1, n = 31) was 16.6% (dotted line) and that of patients in the high AgNOR group (AgNOR score ≥ 6.1, n = 18) was 20% (solid line). **Right:** Patients were divided into two groups according to the AgNOR score of the metastatic lymph nodes. The 5-year survival rate of patients in the low AgNOR group (AgNOR score <5.3, n = 30) was 16.7% (dotted line) and that of patients in the high AgNOR group (AgNOR score ≥ 5.3, n = 19) was 21.6% (solid line).

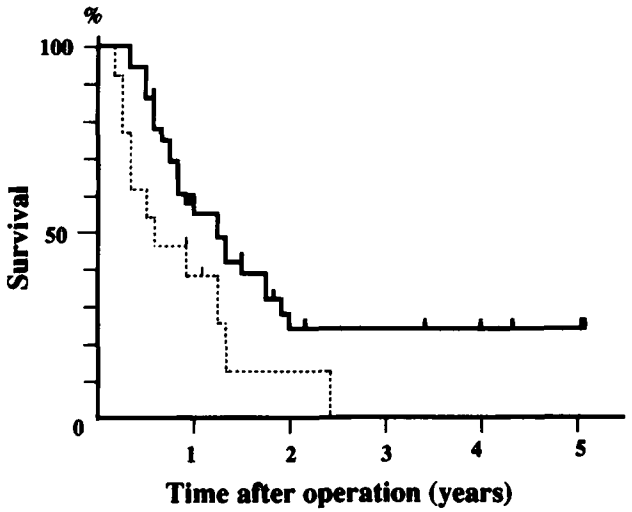

Fig. 4. When the AgNOR scores of metastatic lymph nodes were compared with those of primary tumors in 53 patients with positive nodes, AgNOR scores of metastatic lymph nodes were found to be higher than those of primary tumors in 14 patients. Eleven of these 14 patients died from cancer recurrence within 3 years after surgery, and the 3-year survival rate of this group was 0% (dotted line). In 39 patients, AgNOR scores of metastatic lymph nodes were lower than those of primary tumors. In these 39 cases, the 5-year survival rate of 36 surviving patients was 23.7% (solid line). There is a statistically significant difference between these two survival curves ($P = 0.036$).

cancer cells of primary tumors and of metastatic lymph nodes in node-positive patients, it was found that AgNOR scores of metastatic lymph nodes were higher than those of primary tumors only in 14 of 53 patients (26.4%). Eleven of these 14 patients died from cancer recurrence within 3 years after surgery, and no patients survived longer than 3 years after surgery. By contrast, AgNOR scores of metastatic lymph nodes were lower than that of primary tumors in 39 of 53 (73.6%) patients and the 5-year survival rate of these patients was 23.7% and some of them were still alive more than 5 years after operation.

Our observations indicate that cancer cells with high proliferative activity have the potential for metastasis. However, the proliferative activity of cancer cells that have metastasized to regional lymph nodes seems to be suppressed. Imai and Yamanaka [17] reported that lymph nodes with metastatic cancer cells contained significantly more dendritic cells, which were positive for S-100 protein and served as immunological defenses against tumor cells, than those without metastatic cancer cells. Thus, there is a possibility that immunological defenses against tumor cells are mounted at the regional lymph nodes and that may play an important role in the host's attempt to protect itself against the tumor. Thus, the proliferative activity of cancer cells in the regional lymph nodes is often suppressed. Cancer cells with higher proliferative activity in the regional lymph nodes than in the primary tumors may overcome such immunological defenses, and further metastasis may occur. As a result, the prognosis in such cases may be poor. So, even though the number of AgNORs in cancer cells (AgNOR score) is not an independent prognostic factor for patients with esophageal cancer, it seems very important to compare the AgNOR scores of primary tumors and those of metastatic lymph nodes in order to estimate the prognosis of patients with esophageal cancer with metastatic lymph nodes.

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